resultant piRNAs. If we can monitor the behavior of each piRNA in cells, we will be able to show whether this pathway actually exists or not. Towards this goal, it will be necessary to develop a system in which piRNAs can be traced individually in their amplification cycle.

SUPPLEMENTAL INFORMATION

Supplemental Information contains one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.001.

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Reply to Shoji and Katsuma

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Our paper reports a novel strategy for the artificial introduction of DNA methylation in mouse gonocytes [1]. The manuscript presents data showing that the concomitant expression of sense and anti-sense of EGFP transgenes in embryonic male germ cells induces gene silencing via the piRNA pathway and that the expression of an antisense Dnmt3L transgene induces silencing of the endogenous Dnmt3L gene.

Based on our study of EGFP transgenic mice, we concluded that the expression of sense and anti-sense EGFP genes is necessary and sufficient for piRNA production. Analyzing the data on piRNA expression from the Miwi2 promoter region of the antisense EGFP transgenic mice, Shoji and Katsuma raised a serious concern regarding this conclusion [2]. Given the data on the piRNAs of the Miwi2 promoter region, we would like to withdraw one of our conclusions, "concomitant expression of sense and antisense RNA transcripts is necessary and sufficient for piRNA production."

We concluded based on gene capture analysis that the transgene was not integrated into a 'typical' piRNA cluster, because the number of piRNAs corresponding to the integrated region was not significant. That is, we adopted a kind of 'prospective' definition of piRNA clusters. Meanwhile, Shoji and Katsuma adopted criteria to define piRNA clusters based on bioinformatic analysis of transgenic mice. For transgenes that produce piRNAs corresponding to their own sequences, the loci into which the transgenes are integrated are interpreted as belonging to the piRNA clusters. We agree with their claim based on this definition; however, at the same time, we consider that a more precise definition of piRNA clusters would be necessary.

There are still some ambiguities to be addressed. Although we did not perform deep sequencing analysis of small RNAs, similar gene silencing by the antisense EGFP transgene was found in two other transgenic lines. These transgenic lines silenced the expression of Oct4–EGFP in the double transgenic mice, in which both sense and antisense EGFP were expressed concomitantly, as well as transgenic line #1. In order to draw a robust conclusion, we will further analyze piRNA production in these two antisense transgenic animals and report the results in the near future.

There are two possible results. In the first case, these transgenic mice may also produce piRNAs corresponding to the MIWI2 promoter and EGFP transgene. If this is the case, we are willing to conclude that they are integrated into 'minor' or 'atypical' piRNA clusters. In this case, comparison of the expression of the amount of piRNA corresponding to the transgene will allow us to determine whether the amount of piRNAs or another factor is necessary for gene silencing via the piRNA pathway. This is because the simple existence of transgene-related piRNAs did not induce gene silencing in antisense transgenic mice (Figure 1C in [1]). In the second case, at least one of the two transgenic mouse lines may not produce a significant amount of piRNAs corresponding to the transgene. This would confirm our initial interpretation.

The main source of our misunderstanding was the discrepancy between artificial piRNA production and gene silencing. During the course of our experiments and publication process, we mistakenly believed that they should be concomitant. This discrepancy raises an interesting and important question about the relationship between piRNA production and gene silencing. Abundant piRNAs and/or piRNAs with some unknown characteristics may be necessary for DNA methylation and subsequent gene silencing. Finally, we would like to emphasize that all of the data presented in our paper and the main conclusion are robust.

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